Short Communication

GTPase Activity in Rice Plasma Membrane
Preparation Enhanced by a Priming
Effector for Plant Defence Reactions

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(Received July 26, 1994;

Accepted February 20, 1995)

INTRODUCTION

Self-defence reactions against blast fungus are induced by a fungal proteoglucomannan elicitor at an early stage of infection in rice plant.¹⁾ Recent studies have revealed the involvement of signal transduction system, including phosphoinositidase C and Ca^{2+} (or calcium modulated protein) dependent protein kinase(s), in the early defence reactions in rice plant, leading to activation of O_2^- forming system and phospholipase A_2 .^{2,3)} In addition to the resistance reactions triggered by the fungal elicitor, some non-antifungal chemical agents, such as 1,2-benzisothiazol-3(2H)-one 1,1-dioxide (BIT), an active form of probenazole, and *N*-cyanomethyl-2-chloroisonicotinamide (NCI) are known to act as priming effectors for acquired systemic resistance in rice plants. While the molecular mechanism by which these priming effectors induce resistance still remains to be elucidated, it has been demonstrated that NCI activates PI turn-over in elicitor-stimulated rice-embryo culture cells.⁴⁾ The result suggests that the molecular target(s) of NCI resides in upstream of the phosphoinositidase C system coupled to the putative receptor for the elicitor, and that the action site of BIT might also be located in these systems. Thus we have focused our attention on the effects of the elicitor and BIT on the phosphoinositidase C system in rice plasma membrane. This paper deals with the preparation of the plasma membrane from rice leaves and the effect of BIT and/or the fungal elicitor on the GTPase activity in the preparation.

MATERIALS AND METHODS

1. Reagents

Salicylhydroxamic acid (SHAM), phenylmethylsulfonyl fluoride (PMSF), 2-(*N*-morpholino)ethanesulfonic acid (MES), dithiothreitol (DTT), tris(hydroxymethyl)aminomethane (Tris), bovine serum albumin (BSA), creatine phosphokinase and nucleoside diphosphate kinase were obtained from Sigma Chemical Co., U.S.A. [γ -³²P]GTP (30 Ci/mmol) was purchased from Amersham Japan. Other reagents used were the highest grade commercially available.

2. Preparation of Rice Plasma Membrane and Blast Fungus Elicitor

In this study, we used the same lot of plasma membrane stock prepared from the rice seedlings grown in a phytotron from May to October. Rice blades at 4 to 5 leaf stage of seedlings (cv. Aichiasahi) were excised and washed with water. The blades (*ca.* 30 g fresh wt.) were cut into fine cross-sections and homogenized in 300 ml of homogenization medium (5 mM EGTA, 5 mM EDTA, 10 mM NaF, 2.5 mM $\text{Na}_2\text{S}_2\text{O}_4$, 1 mM DTT, 4 mM SHAM, 2 mM PMSF, 0.5% BSA, 0.3 M sucrose, and 50 mM MES-Tris; pH 7.6) using a Nissei AM-8 homogenizer for 1 min for 3 times at 4°C. The homogenate was filtered through a double-gauze and 80 μm nylon-meshes. The filtrate was centrifuged at $10,000 \times g$ for 30 min, and an equal volume of suspension medium (0.25 M sucrose and 10 mM $\text{KH}_2\text{PO}_4/\text{K}_2\text{HPO}_4$; pH 7.8) was added to the supernatant. The mixture was ultracentrifuged ($100,000 \times g$, 30 min) to obtain the sediment of microsomal fraction and the precipitate was resuspended in 6 ml of the suspension medium. The plasma membrane fraction was purified from the crude suspension by the aqueous two-phase partition according to the procedures reported⁵⁻⁸⁾ with some modifications as follows. The mixture of 6% (w/w) of each Dextran T-500 and polyethylene glycol 4000 in the suspension medium was added to 1 ml of the suspension of crude plasma membrane to give a total weight of 9 g. Then NaCl was gradually added to the mixture with gentle stirring to make the concentration from 20 to 60 mM, and the resultant mixture was centrifuged at $1500 \times g$ for 5 min. To the upper phase, 20 ml of isolation medium (10 mM NaF, 2.5 mM $\text{Na}_2\text{S}_2\text{O}_4$, 1 mM DTT, 2 mM PMSF, 0.3 M sucrose,

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and 50 mM MES-Tris; pH 7.6) was added and shaken. Following ultracentrifugation at $100,000 \times g$ for 60 min, the pellets were resuspended in 2 ml of the diluted isolation medium (10-folds with deionized water) and stored at -80°C , if necessary. The stocked membrane preparation was used for the experiment within a week.

The preparation of proteoglucomannan elicitor (25 kDa fragment) from blast fungus (H 373: race 007) was performed according to Kanoh *et al.*²⁾

3. Assay of Plasma Membrane Preparation

The micro-assays of inorganic orthophosphate (Pi) and total protein were done according to Heinonen & Lahti⁹⁾ and Bradford,¹⁰⁾ respectively. Purity of plasma membrane preparation was assessed by measuring the activity of marker enzymes, *i.e.*, Na_3VO_4 sensitive ATPase, inosine diphosphatase (IDPase) and NADH-cyt. c reductase as described previously.^{7,11)}

4. Determination of GTPase Activity

Determination of rice plasma membrane GTPase activity was achieved according to Shenker *et al.*,¹²⁾ Aktories & Jakobs,¹³⁾ and Cassel & Selinger¹⁴⁾ with some modifications. The assay system contained $0.67 \mu\text{M}$ [$\gamma\text{-}^{32}\text{P}$]GTP (4.4×10^6 dpm), 0.1 mM EGTA, 2 mM MgCl_2 , 1 mM DTT, 0.1 mM ATP, 5 mM phosphocreatine (Tris salt), 10 units creatine phosphokinase, and 10 units nucleoside diphosphate kinase in 50 mM BisTris-HCl (pH 7.0). The final volume was 100 μl , and sodium deoxycholate (DOC, 0.05% w/v), BIT (1 mM) and/or blast fungus elicitor (5 μg) were also included in the reaction mixture when necessary. Reactions were initiated by the addition of rice plasma membrane preparation (eq. to 25 μg plasma membrane protein) to the mixture at 25°C and were terminated by the addition of 750 μl of cold 5% (w/v) activated charcoal in 20 mM phosphoric acid. Tubes were fully shaken and centrifuged ($6000 \times g$ for 20 min at 4°C) and ^{32}P in 425 μl of each supernatant was counted in a scintillation spectrometer using Cerenkov radiation.¹²⁾

RESULTS AND DISCUSSION

Preparation of rice plasma membrane were carried out by the two-phase partitioning method, fundamentally following the procedures developed for orchard grass (*Dactylis glomerata* L.),⁵⁾ mung bean (*Vigna radiata* L.),⁶⁾ and corn (*Zea mays* L.).⁷⁾ However, the optimal concen-

Fig. 1 Relationships between membrane-bound enzymes and sodium chloride concentration in the partition mixture.

●: Na_3VO_4 -sensitive ATPase, ○: IDPase, ■: NADH-cyt. c reductase.

Table 1 Effects of inhibitors on Mg^{2+} -ATPase in the plasma membrane preparation.

Addition (mM)	Mg^{2+} -ATPase activity (%) ^{a)}	
	NaCl: 50 mM ^{b)}	NaCl: 60 mM ^{b)}
KNO_3 (50 mM)	14	6
NaN_3 (1 mM)	10	8
Na_3VO_4 (0.1 mM)	84	86

^{a)} Figures indicate inhibition % by inhibitors on Mg^{2+} -ATPases in rice-blade plasma membrane preparation.

^{b)} NaCl concentrations used for preparation of rice plasma membrane fraction.

tration of NaCl in the partition system appeared to differ significantly depending on plant species used for the experiments. In contrast to the membrane preparation described in the previous papers,^{5,6)} a high concentration of NaCl (50–60 mM) gave the best separation profile of the plasma membrane fraction in rice plant as assessed by the distribution of marker enzymes (Fig. 1). Only low contamination of Golgi membranes with IDPase activity was observed and negligible NADH-cyt. c reductase activity specific for ER was detected. The ATPase activity in the preparation was characterized by

Fig. 2 Effects of BIT and blast fungus proteoglucomannan elicitor on GTPase activity in rice-blade plasma membrane preparation.

□: PM without DOC, ■: PM with DOC, △: PM+DOC+elicitor, ○: PM+DOC+BIT, ●: PM+DOC+BIT+elicitor. PM: rice-blade plasma membrane preparation, DOC: sodium deoxycholate, BIT: 1, 2-benzisothiazol-3(2*H*)-one 1, 1-dioxide. The incubations were conducted at 25°C for each indicated time. Bars indicate standard error in duplicated measurements.

determining its sensitivity to specific inhibitors, which showed quite low contamination of membranes of tonoplasts (sensitive to KNO_3) and mitochondria (sensitive to NaN_3), as shown in Table 1. These results demonstrate the isolated plasma membrane fraction to be suitable for further biochemical analyses.

Figure 2 shows the stimulation of GTPase activity in plasma membrane by the fungal elicitor and/or BIT. In either case, an immediate increase of Pi release was observed (0–1 min) after initiation of the reaction. Following the constant state lasting for 3 min (1–4 min), an abrupt reevaluation of GTPase activity was observed (4–5 min). The level of elevation was highest in the presence of both elicitor and BIT (A_0 – A_1 in Fig. 2), but the amount of released Pi gradually decreased after A_1 . The apparent downwards regulation (A_1 – B_0) may be attributed to the function of putative H^+ translocating ATPase, Ca^{2+} – H^+ antiporter, or Ca^{2+} translocating ATPase existing in the rice plasma membranes, though the precise explanation is not available at present. However, such profiles as shown in

Fig. 2 were repeatedly obtained under conditions described above.

The GTPase reached an activated state quite effectively in response to the existence of BIT, a priming effector for acquired systemic resistance in rice plant, which supports our hypothesis that the activation of putative G-protein may be involved in the signaling pathway leading to earlier biochemical defence reactions. Naim *et al.* recently reported that amphiphilic sweeteners, such as aspartame and soluble saccharin (sodium salt of BIT) directly activate G_1/G_0 -proteins and transducin, which belong to the same G-protein superfamily in mammalian systems.¹⁵⁾ Our present study suggests that BIT may function as G-protein activators in rice plants as well. The hypothesis should be confirmed by the future G-protein binding assays using the plasma membrane preparations isolated from rice blades by the procedures described.

In summary, the rice plasma membrane fraction prepared by the two-phase partitioning method proved to exhibit GTPase activity which

is stimulated by blast fungus proteoglucomannan and/or BIT, a priming effector, in the presence of DOC. The result suggests that BIT enhances the potential plant defence abilities, either directly or indirectly, through activation of putative G-proteins.

ACKNOWLEDGMENTS

We thank Drs. Daijiro Hosokawa and Tohru Teraoka, Tokyo University of Agriculture and Technology for their invaluable support and discussion throughout the study, and to Ms. Shigeko Sekido in our lab. for her skillful supports to maintain rice seedlings in phytotron. The research was in part supported by the grant for "Biodesign Research Program" from The Institute of Physical and Chemical Research (RIKEN).

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要 約

病害抵抗性付与剤によるイネ葉細胞形質標品 GTP アーゼ活性の亢進

関沢泰治, 青山秀幸, 木村 真, 山口 勇
イネ葉細胞のミクロソーム画分をポリマー二相分離法で分別し, 他の膜成分の混入の少ない形質膜標品を得た. この形質膜標品にイネいもち病菌由来のプロテオグルコマンナン エリシターを加えることにより, 膜結合 GTP アーゼの活性増大が認められた. またプロベナゾールの活性代謝物 BIT の存在下で, このエリシターの効果は顕著に高進した. これらの結果は, BIT 等の全身獲得抵抗性付与剤による病害抵抗性の早期成立に関わる GTP 結合タンパク質が, イネ葉細胞の膜情報伝達系に存在することを示唆している.